THE EFFECTS OF THIOUREA AND SOME RELATED COMPOUNDS ON REGENERATION IN PLANARIANS[1]

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During recent years much research has been devoted to the effects of the administration of various anti-thyroid agents to vertebrate animals. Interest is due to the fact that these agents have been demonstrated to inhibit the activity of the thyroid gland. Only a few studies have been made of the effects of such drugs on invertebrates, and the majority of these deal with the effects of the goitrogens on fertilized eggs and developing embryos. Bevelander (1946), using fertilized sea urchin eggs placed in test solutions of 0.1–1.0% thiourea in sea water, found that at a concentration of 1.0% no cleavage occurred, but cleavage was normal in a similar concentration of urea, indicating the inhibition of cleavage was not due to any osmotic effect. Lower concentrations produced a retardation in over-all growth rate. Rulon (1950), studying the modifications in developmental patterns in the sand dollar by thiourea, reports substantially similar results.

The present investigation was undertaken in order to study some comparative effects of varying concentrations of thiourea and related compounds on an invertebrate beyond the embryonic stage. For this study a species of planarian, a freshwater flatworm, was chosen. In this animal, when the tail is separated from the body by a dorso-ventral cut posterior to the pharynx, the body will produce a new tail, and the separated tail will regenerate all missing structures, becoming a new and independent organism. A study was made of the rate of growth of a new tail by the body, and of the time required for the appearance and development of the regenerated organs in the newly formed worm. Observations were also made of any modifications in the regenerating structures, due to the action of the goitrogens, and of pigment loss or lack of development, both in the new tissue and in the old, mature cells.

Materials and Methods

The animals used in this study were specimens of *Dugesia tigrina*, collected in a stream near Baltimore, Maryland. Stock animals were fed once a week. Experimental animals were taken five days after feeding, and were not fed during the experiment.

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Three chemicals were used in the study: thiourea, phenylthiourea, and thiouracil. Since a comparative study of the effects was to be made, three series of experiments were performed, using concentrations of 0.005%, 0.01%, and 0.02% of each chemical. Worms chosen for experimentation were as near the same size as possible, varying from seven to nine millimeters in length. Tails were severed a short distance behind the pharynx, and placed in fingerbowls of the proper solution. These were stacked to prevent evaporation. The bodies were placed similarly in other fingerbowls. The worms were handled with sable-hair brushes, or wide-tipped medicine droppers. Control animals were kept in tap water.

In Series I, the experimental animals were placed in 0.005% solutions of the chemicals. No observations were made on the regenerating tails the first day after cutting. Beginning with the second day, the tails were observed every day for ten days, then on the fourteenth, eighteenth, and twenty-fifth days. At the end of seven days, the worms in each chemical were divided into two groups. One group was kept in the chemical until the end of the experiment; the other group was returned to water to see if any of the effects noted were reversible.

For observation, the tails were placed in a drop of the solution on a microscope slide, and observed through the low-power objective of a compound microscope, using a blue filter in a standard lamp. Information was obtained concerning the time in days required for healing to take place, and for eyes, proboscis, and sense lobes to form. Observations were also made concerning the color and appearance of the eyes and of the proboscis, and of such noticeable special effects as might occur.

The bodies of the worms were observed every second day for the first week, and every third day thereafter. At the end of seven days the worms in each chemical were divided into two groups. One group was returned to water; the other remained exposed to the goitrogen. The rate of regeneration was observed by measuring the lengths of the worms on successive days. The effect of the chemicals on the pigmentation was noted.

For measuring, a somewhat modified form of the method originated by Wulzen (1927) was employed, and the average length of worms in each solution was computed. Graphs of growth rate were made, plotting average lengths, calculated to 0.1 mm., against time in days. In order that a better comparison of growth rates in the different solutions and series might be made, the daily average length in each group was recalculated, using as the original average length on the day of cutting that average exhibited by the water controls, namely, 5.6 units.

A second series of experiments, using a concentration of 0.01% of each of the chemicals, was performed. No other change was made in either method or materials. A third series, using a 0.02% concentration was likewise performed, but due to the toxicity of phenylthiourea at this concentration, a comparative study of effects at correspondingly higher concentrations was not attempted.

The Effect of the Goitrogens on Severed Tails

Healing. In the normal planarian, when a tail is severed, the cut edge contracts, forming a pronounced, black indentation, semi-circular in shape. Within two to three days, as healing progresses, relaxation occurs, and the newly forming, unpigmented flesh is protruded forward in a more or less triangular shape as the worm glides about. In the worms treated with chemicals, the healing process was notice-

ably slowed. When thiourea was used, the effect appeared to be in proportion to the concentration used. Worms placed in a 0.005% solution were all healed on the third day, in a 0.01% solution on the fourth day, and in a 0.02% solution on the fifth day.

The phenylthiourea was markedly more effective than the thiourea, even in the lower concentrations. It was not until the sixth day that healing occurred in all worms placed in a 0.005% solution, and in a 0.02% solution complete healing did not occur. The effect of the thiouracil solutions on healing, while greater than that of the thiourea, was less than that of the phenylthiourea. Worms placed in a 0.005% and in a 0.01% solution were healed by the third day, but six days were required for complete healing of those placed in the 0.02% solution.

Formation of sense lobes. When a head is forming in a regenerating planarian, by the fourth or fifth day the triangular protuberance of unpigmented new flesh has become sufficiently large that the animal, in moving about, exhibits the beginnings of sense lobes by protruding and withdrawing, seemingly at will, a small bit of tissue on either side, just anterior to the healed cut. In this experiment, it was found that the 0.005% solution of each of the three chemicals and the 0.01% concentration of thiourea and thiouracil were ineffective in retarding this. All the animals in these solutions were able to produce sense lobes by the fifth day.

The other concentrations used were more effective in this respect. Sense lobes appeared in all worms in the 0.02% solutions of thiourea and thiouracil on the sixth day, and in the 0.01% concentration of phenylthiourea on the eighth day. It was not until the tenth day, however, that the worms in 0.02% phenylthiourea showed this stage of development. In the worms returned to water from higher concentrations of the chemicals, the sense lobes appeared within twenty-four hours after return, or by the eighth day.

Proboscis development. The first definite sign of a developing proboscis in a severed tail can be seen in a freely moving planarian on the third or fourth day after cutting. A smooth, tan-colored protuberance appears at the point where the two sides of the digestive tract have grown together, and grows caudally until its length is about four times its width. Pigmentation and wrinkling, the latter due to an increase in real but not apparent length, occur on the fifth or sixth day after cutting, in the normal worm.

In this experiment both the 0.005% and the 0.01% solution of each of the three chemicals had little effect on the time required for the appearance of the proboscis, or on its subsequent development, but each of the chemicals was effective at a concentration of 0.02%. At this concentration the organ could be seen in all the animals in thiourea and thiouracil on the fourth day, but it was not until the fifth day that it could be found in all of the worms in phenylthiourea. Further development of the proboscis was also affected. By the fourteenth day the worms in both thiourea and phenylthiourea exhibited a very immature proboscis, shorter and narrower than is normally found on the fourth day. The latter solution was particularly toxic. The animals in thiouracil fared better. In them the proboscis, while less mature in appearance than those in the water controls, was apparently able to function normally. The effect was reversible in the worms returned to water at the end of seven days. In these worms, by the fourteenth day the proboscis was as developed, pigmented, and wrinkled as those of the water controls.

Eye formation. Eye formation in the normally regenerating planarian begins

quite early. By the third day definite, tiny, black eyespots can be seen under the low power of the microscope, and by the sixth day the spots have become large and black, curved and smooth in outline on the median side, and concave and slightly granular on the lateral side.

In this experiment the effect of the thiourea was quite varied as far as individual worms were concerned, but the concentration did not seem to cause a marked difference. At all three concentrations the developing eyes were somewhat smaller and more granular in appearance than those of the water controls. The black pigment that formed began to disappear irregularly on the sixth day in Series I and II, and on the fifth day in Series III. On the seventh day, before the transference of half the animals to water was made, it could be seen the pigment was disappearing to a greater or lesser extent in the eyes of all the worms at all three concentrations. During the following week a change could be noted daily. All the worms which were kept in the 0.005% solution of thiourea lost all eye-pigment by the eighteenth day. The animals in the 0.02% solution of thiourea lost all eye-pigment by the tenth day of subjection to the chemical, but in each one there persisted a distinct, ghost-like outline of the eye shape, very faintly yellowish-pink in color. The 0.01% concentration was variable in its effects. By the twenty-fifth day, in one of the worms there was a nearly normal amount of black pigment, while in the others the pigment was nearly gone, but in no case was it completely absent. In contrast, the worms which were returned to water gained pigment little by little, until by the fourteenth day they closely resembled the water controls.

The phenylthiourea, at all concentrations used, inhibited pigment formation completely in the developing eyes, although the eyes themselves could be seen in faint, ghost-like outline, faintly yellowish-pink in color. In Series I, the eyes of the worms which remained in the chemical showed during the second week a faintly brown, smooth outline. By the eighteenth day this was more pronounced, and by the twenty-fifth day reddish-tan granules had begun to appear in the eyes. It is possible that black pigment might have eventually developed, but the regenerating tails, which had been without food over three weeks, had become so small that sustenance was necessary for their continued existence, and the experiment was brought to a finish.

In both the two higher concentrations of phenylthiourea, the worms which remained in the chemical during the entire experiment showed practically the same effect. After the eye outlines appeared, there was no change until the fourteenth day, when a slightly pinker color began to show. In the worms in Series II, the eyes were full size and very pink in color on the twenty-fifth day, but the worms in Series III had died and disintegrated by the eighteenth day, so that further observation was impossible.

In the worms which were returned to water from each of the three concentrations of phenylthiourea, a steady development of pigment followed. The smooth outline became darker and a golden-brown color developed inside. This gradually changed to a reddish-brown, then black. The eye outlines became granular as the darker colors appeared. By the fourteenth day, the eyes of all returned to water appeared like the eyes of the water controls, with the exception that these retained a slightly reddish cast. By the twenty-fifth day these were indistinguishable from the water controls.

Solutions of thiouracil showed much less effect than solutions of either thiourea or phenylthiourea. In all cases the general effect of the chemical was to cause the eyes to become slightly more granular in appearance than is normal, and to become slightly reddish in spots as the pigment partially disappeared. This was more pronounced in the higher concentrations, but in no case did the pigment completely disappear, even after twenty-five days exposure to the chemical. Worms returned to water on the seventh day regained normal eye appearance within three days.

Skin pigmentation. During all series careful attention was given to possible effects of the chemicals on skin pigmentation, both in mature cells and in newly forming tissue. No bleaching effect was noticed under the influence of any one of the three chemicals, at any concentration used, up to twenty-five days, when the experiment was terminated.

The Effect of the Goitrogens on Growth Rate

By a comparison of the average lengths of the worms, as measured on succeeding days, it was found that regenerating planarians in water, at a controlled temperature, exhibit a characteristic growth curve. For the first four days after the tails are severed, rapid growth of the bodies occurs, followed by two days of slower growth. The maximum length is reached on the sixth day. Following this, if food is not given the animal, it must begin to live on its own tissues, and a decrease in length results. After a four- to five-day interval, the graph line begins to level off somewhat. Another period of rapid decline follows, then another period of levelling-off.

The characteristic growth curve of planarians in water is shown in Figure 1, together with a typical response of the animals to the effects of the goitrogens. In this graph, the regenerative growth rate of worms subjected to a 0.02% solution of thiourea, and of those returned to water at the end of seven days, is compared with the curve exhibited by the water controls. It will be noticed the peak of growth occurs on the sixth day for both groups of animals, although the peak attained by the experimentals is lower. The graph line for the planarians returned to water shows the characteristic lessening of retardation of growth. A study of the comparative effects of thiourea at different concentrations reveals that the 0.005% concentration is least effective in depressing the growth rate, and recovery from exposure to it follows most rapidly; the 0.01% solution is most effective in depressing the growth rate during the first few days of exposure; and the 0.02% concentration, while not most effective in depressing the initial growth rate, is much more potent after long exposure.

The distinct lessening of retardation of growth in animals returned to water at the end of seven days was quite apparent in all three series with each chemical used. In the majority of cases the lessening of effect was so pronounced that a second growth peak was reached. This was especially noticeable in animals exposed to thiouracil. In Figure 2 the second growth peak is shown to have occurred on the fourteenth day, or seven days after the planarians were returned to water from 0.02% thiouracil. The occurrence of the second growth peaks ranged from the eleventh to the fourteenth day.

It was found by a comparison of the effects produced by each of the goitrogens at a concentration of 0.005% that the thiourea affected the rate of growth less at this concentration than did either thiouracil or phenylthiourea, and that the latter

was the most effective. This conforms with the findings above of the influence of the chemicals on the regeneration of missing organs in severed tails.

A study of the growth rate of planarians in a 0.01% solution of the chemicals showed that, while initial exposure to thiourea at this concentration was not highly effective, continued exposure produced a marked retardation in growth, and a return to water allowed nearly normal growth to be resumed. At this concentra-

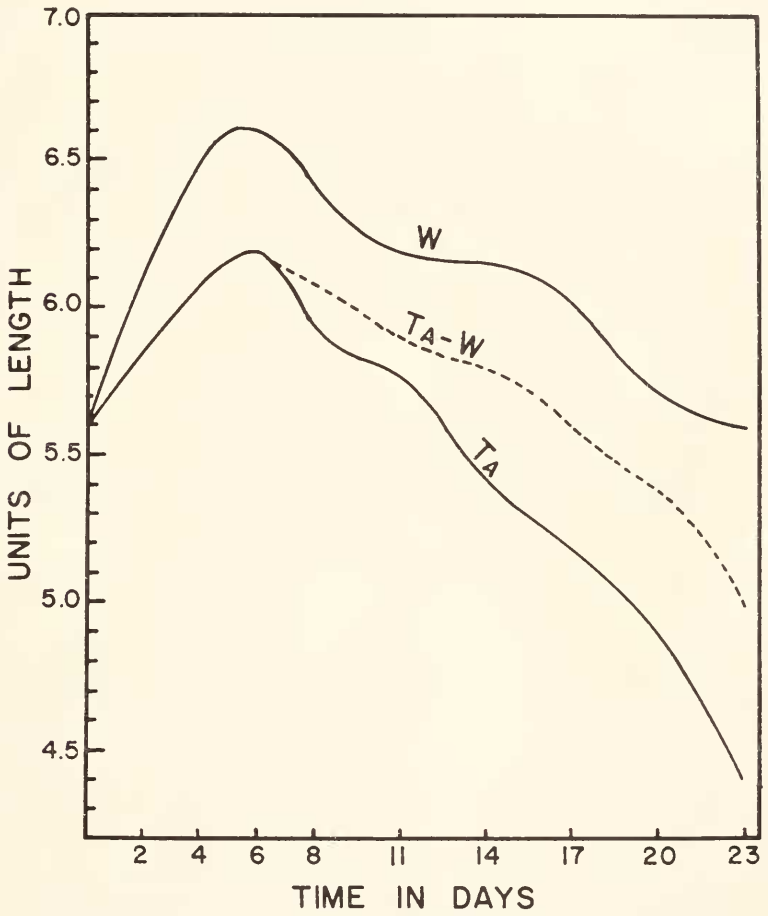

FIGURE 1. Growth rate curve of planarians exposed to 0.02% thiourea (Ta) and of those returned to water at the end of seven days (Ta-W) compared with the characteristic curve of water controls (W).

tion both phenylthiourea and thiouracil were found to be quite effective in depressing initial growth, so much so that the peak of growth was not only quite low, but was reached seven to eight days after exposure to the chemical, or one to two days later than the peak observed in the water controls.

A comparison of the effects of exposing the experimental animals to a 0.02%

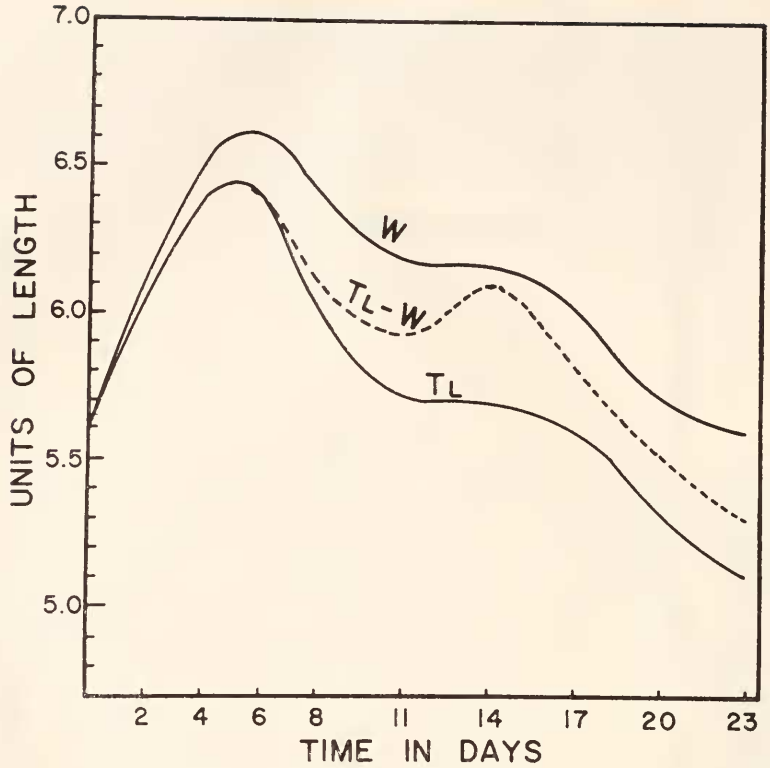

FIGURE 2. Growth rate curve of planarians exposed to 0.02% thiouracil (TL) and of those returned to water at the end of seven days (TL-W) compared with the characteristic curve of water controls (W).

concentration of the chemicals revealed that, at this concentration, thiouracil was least effective in retarding growth, while phenylthiourea was most effective. A marked depression, approaching toxicity, followed continued exposure to both thiourea and phenylthiourea, but a noticeable recovery was made when the animals were returned to water after a week's exposure. This, too, is in accord with the facts noted above.

DISCUSSION

In the present study, it was found that the normal regenerative powers of the planarians were reduced by the administration of goitrogenic agents in varying concentrations, and that the effect was more pronounced as the concentration was increased. This is in conformity with the findings of Lynn (1948) and Rulon (1950). Lynn, testing two of the thioureas on a toad, *Eleutherodactylus ricordii,* which possesses no aquatic larval stage, found that a concentration of 0.001% thiourea was ineffective, a concentration of 0.005% was slightly effective, and that a concentration of 0.05% thiourea caused a definite retardation in development. Rulon reported that continuous exposure of newly fertilized eggs of *Dendraster* to low concentrations of thiourea resulted in slight inhibition of development, and that with higher concentrations the degree of inhibition increased.

In this experiment the depression of growth rate by the goitrogens, noticeable to some extent at all concentrations, was shown not only by the lower peak of growth as exhibited by the graphs, but also by the fact that certain of the concentrations slowed the initial growth sufficiently that the peak was reached after seven to eight days' exposure to the chemical, at a time when the period of rapid decline was apparent in the water controls. A possible explanation of this is that the lowered metabolic rate allowed a longer use of the food present in the animal, before the necessity of subsisting on its own tissues became imperative.

The second growth peaks noted in the majority of animals returned to water, which occurred at a time when a levelling-off period was to be found in the water controls, were apparently due to an upsurge of metabolic activity following the release of the animals from the influence of the goitrogens. This effect appears to be similar to that noted in the severed tails, when rapid reconstitution of deficient organs followed the return of the animals to water.

None of the chemicals used had any appreciable effect on head formation, the appearance of functioning sense lobes, or the development of the proboscis, when used at a concentration of 0.005%, and only phenylthiourea exhibited a marked modifying action at a concentration of 0.01%. All three chemicals, at a concentration of 0.02%, produced a distinct retardation in all phases of organ development. The results of this study show that not only is the retardation of the metabolic rate of planarians, as evidenced by the rate of regeneration, influenced by the degree of concentration to which the animals are subjected, but that certain goitrogens are more effective than others in this respect. In all phases of the study, phenylthiourea was found to be more potent in repressing the rate of regeneration, and in causing modifications in developing organs, than either thiourea or thiouracil. This, too, is in agreement with the results obtained by Lynn (1948), who found that a 0.005% concentration of phenylthiourea was as effective in retarding embryonic development as was the 0.05% thiourea.

Reports of several workers indicate that the development of pigmentation in the animal body is intimately associated with the metabolic process. Lynn (1948), treating leptodactylid embryos with 0.005% phenylthiourea, found that not only was there a definite retardation in development, but that within three days the experimentals were noticeably lighter than the controls, and by the sixth day all visible dark pigment, both in the skin and in the retina of the eye, had disappeared. Frieders (1954), studying the effect of the same chemical on fish, found that the animals showed a definite loss of body pigment, and that a gradual but noticeable loss of pigment could be observed in the eyes. At the same time, the growth rate of the experimentals was much slower than that of the controls.

While no bleaching effect in regard to skin pigmentation was noted at any time in this experiment, it was found that all three chemicals interfered to some extent with the production of eye-pigment at all concentrations, the effect increasing as the concentration was increased. That the goitrogens inhibited pigment formation, not the development of the eye itself, was shown by the fact that the planarians, particularly those in phenylthiourea, developed eye outlines, although pigment did not appear.

In this study, as in those cited above, the rate of metabolism of the planarians, as evidenced by the growth rate and by the appearance of new organs appeared to parallel the speed or slowness of pigment formation. It is probable that a funda-

mental correlation exists between the production of animal pigment and the production of chemicals which exert a controlling influence on the metabolic rate. The fact that goitrogens affect metabolism and pigment formation similarly in both vertebrates and invertebrates lends support to this view.

Summary

1. A study was made of the effects of the three goitrogens, thiourea, phenylthiourea, and thiouracil, on *Dugesia tigrina*, a species of planarian. Observations were made of the effects of the drugs on healing, head formation, proboscis development, eye and skin pigmentation, and regenerative growth rate.

2. Phenylthiourea was found to be most effective in preventing healing. Both thiourea and thiouracil retarded the rate of healing.

3. Higher concentrations of all three goitrogens were effective in retarding or suppressing the normal development of sense lobes and proboscis. Phenylthiourea was most potent. Lower concentrations were ineffective. The effect was reversible.

4. Phenylthiourea inhibited eye-pigment formation, but not eye formation. The effect was reversible. Thiouracil had little effect on the formation of eye-pigment. The effect of thiourea was varied.

5. Bodies with severed tails, placed in water, showed a characteristic growth curve when body length was plotted against time in days. Plotted curves of planarians in goitrogens, compared with controls, showed retardation of growth. Noticeable recovery was made upon the return of the experimentals to water.

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